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Cell Wall Assembly in *Bacillus megaterium*: Incorporation of New Peptidoglycan by a Monomer Addition Process

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The pattern of cross-linking in the peptidoglycan of *Bacillus megaterium* has been studied by the pulsed addition of radiolabeled diaminopimelic acid. The distribution of label in muropeptides, generated by digestion with *Chalaropsis* muramidase and separated by high-performance liquid chromatography, stabilized after 0.15 of a generation time. The proportion of label in the acceptor and donor positions of isolated muropeptide dimers stabilized over the same period of time. The results have led to the formulation a new model for the assembly of peptidoglycan into the cylindrical wall of *B. megaterium* by a monomer addition process. Single nascent glycan peptide strands form cross-linkages only with material at the inner surface of the wall. Maturation is a direct consequence of subsequent incorporation of further new glycan peptide strands, and there is no secondary cross-linking process. The initial distribution of muropeptides is constant. It follows that the final pattern of cross-linking in the wall is determined solely by, and can be forecast from, this repetitive pattern of incorporation. In a modified form, this model can also be applied to assembly of cell walls in rod-shaped gram-negative bacteria.

The cell wall in gram-positive bacteria is a thick, elastic structure, closely apposed to the cytoplasmic membrane. The wall serves to protect the underlying protoplast and to prevent the cell from bursting because of the unrestricted inflow of water that would otherwise result from the high internal osmolarity of the bacterium. The strength and integrity of the wall are determined by a complex of peptidoglycan and anionic polymer (teichoic acid or teichuronic acid). These polymers, which are synthesized by membrane-bound enzyme systems, are covalently attached to each other and then incorporated into the wall. The biosynthesis of the individual polymers is now well understood, and studies of various *Bacillus* species have shown that teichoic acid and peptidoglycan are separately assembled on lipid carrier and linked together during, or immediately before, incorporation into the wall. Incorporation involves the formation of peptide linkages between newly synthesised peptidoglycan and material already in the wall, and it takes place apparently while the incoming peptidoglycan is still growing at its lipid anchor in the membrane (3).

In *Bacillus megaterium*, as in most other bacilli, the peptidoglycan has the chemotype A1 in which the peptide chains contain D- and L-alanine, D-glutamic acid, and meso-diaminopimelic acid (DAP). These peptide chains are thought to project from a helically twisted glycan chain (4). During wall assembly, these peptide chains become cross-linked to each other by transpeptidation reactions in which the terminal D-alanine is removed from one of the peptide chains (the donor) while its penultimate D-alanine becomes linked to the ϵ amino group of a DAP in a peptide chain (the acceptor) already in the wall. Where two muropeptide monomers are cross-linked to form a dimer, the original acceptor and donor peptides in the transpeptidation reaction can be identified. The peptide in the donor position of a dimer still has a DAP with an available amino group and is therefore capable of acting as an acceptor to form a trimer. The direction of transpeptidation has been shown to be the

same in *B. megaterium* (18), *Bacillus licheniformis* (35), *Salmonella typhimurium* (9), and *Escherichia coli* (20).

Studies on both gram-positive and gram-negative organisms have shown that the degree of cross-linking of new peptidoglycan increases after its initial incorporation. Early experiments with *Staphylococcus aureus* showed that uncross-linked peptides in newly incorporated peptidoglycan were later chased into higher oligomers (33), and increases in cross-linking after incorporation have also been reported in *B. megaterium* (16), *Streptococcus faecalis* (12), and *E. coli* (11). The enzymes responsible for this secondary cross-linking, or maturation, process are thought to be distinct from those responsible for the initial incorporation (11). Changes in cross-linking following incorporation are an essential feature of current models for peptidoglycan assembly in gram-negative bacteria in which incorporation involves the insertion of new strands by the breakage and reformation of cross-linkages (6, 20).

Wall assembly in gram-positive bacilli can be separated into two distinct processes. Septa, and hence cell poles, are formed by incorporation of new wall material from an annular growth zone, whereas cylindrical wall is formed by incorporation along the entire length of its inner surface (1, 27, 30), which gradually pushes older wall towards the outer surface of the cell. During its progress through the wall, a peptidoglycan layer will become stretched as the cell elongates (13), and this stretching may facilitate the action of autolysins in the outer layers of the wall (25). Since the innermost layers are unlikely to be stress bearing, there is not the same requirement to maintain full mechanical strength during the assembly process as is apparently necessary in gram-negative bacteria.

We have recently demonstrated a protocol for pulse-labeling *B. megaterium* with a defined pulse of DAP (17). In this paper, we describe the use of this technique to study both the location (donor or acceptor) of DAP incorporated into dimers in the wall and the distribution of labeled DAP in the muropeptides at intervals following the pulse. The results suggest a model for the incorporation of single strands of peptidoglycan into the wall in which the formation of

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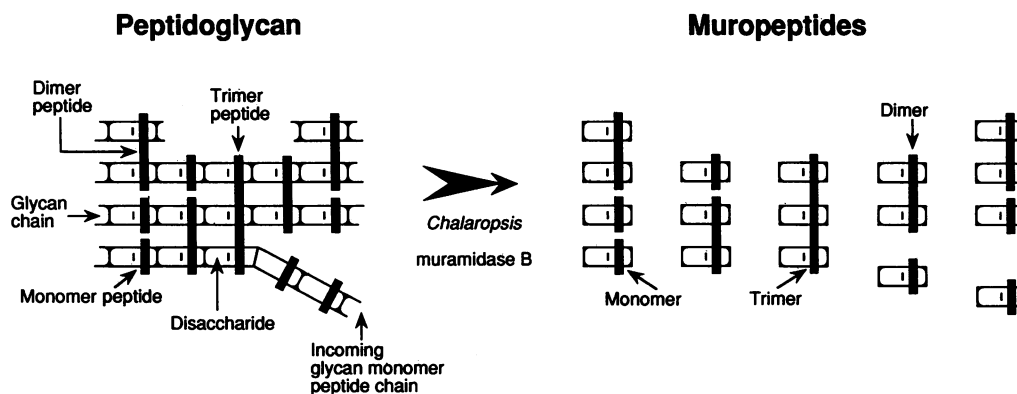


FIG. 1. Schematic diagram showing how the various muropeptides are generated from peptidoglycan by digestion with *Chalaropsis muramidase B*. The terminology is that used throughout the paper.

cross-linkages is solely by the addition of the new peptide monomers to preexisting wall. The model allows for steric constraints in cross-linking and does not require either the concerted action of lytic and synthetic enzymes or a secondary cross-linking process. Variations of this model are also consistent with current information on peptidoglycan assembly in gram-negative bacteria.

MATERIALS AND METHODS

Organism and growth conditions. *B. megaterium* KM (NCIB9521) was grown in 250-ml Erlenmeyer flasks with vigorous aeration at 37°C. The medium was that described previously (17). Growth was followed by monitoring optical density at 600 nm; during exponential growth, the mean generation time was 41.5 min.

Pulse-labeling of peptidoglycan. Cells were labeled by the addition of [³H]DAP [(DL + *meso*)-2,6-diamino (G-³H) pimelic acid dihydrochloride; Amersham International]. A 1-ml solution of labeled DAP and L-lysine was added to 30 ml of an exponential-phase culture (optical density at 600 nm, ~0.3) to give final concentrations of 1 µg of DAP per ml (specific activity, 140 µCi/µmol) and 4 µg of L-lysine per ml. Labeling was terminated by rapid filtration through a prewarmed Millipore filter (0.45-µm pore size), followed by two washes with prewarmed medium (10 ml) and resuspension in prewarmed fresh medium (30 ml). Under these conditions, there was no observable lag and the cells grew at the same rate as before filtration. For the shortest labeling time, filtration and resuspension was unnecessary and 30 s (~0.01 generation time) after the addition of [³H]DAP the culture was added directly to boiling sodium dodecyl sulfate (SDS).

Preparation of cell wall peptidoglycan. Samples of culture (15 ml) were added to 30 ml of boiling SDS (6% [wt/vol] in water) and boiled for 30 min. Insoluble material was recovered by centrifugation (48,000 × g, 40°C, 15 min), resuspended in water, and again boiled in SDS (4% [wt/vol]) for 20 min. The resulting wall preparation was then washed at least four times with hot (90 to 95°C) water. Covalently attached protein was removed by treatment with pronase (type XIV from *Streptomyces griseus*; Sigma Chemical Co.), pre-treated at 60°C to remove contaminating mureinolytic activity (22). The cell wall preparation was suspended in 50 mM Tris chloride, pH 7.0 (1 mg/ml), the pronase was added to a final concentration of 100 µg/ml, and the mixture was incubated for 1 h at 60°C. The walls that were recovered by centrifugation (48,000 × g, 20°C, 15 min) contained 1.6%

phosphorus (7) due to the presence of anionic polymers. These polymers were removed by treatment with aqueous hydrofluoric acid (24). Hydrofluoric acid (0.1 ml of a 48% [wt/vol] solution) was added to the dried cell wall in a 1.5-ml microcentrifuge tube, and the mixture was incubated at 2°C for 24 h. The mixture was cooled to -70°C, and the pH was adjusted to just below 7.0 by the addition of 2 M potassium hydroxide. The peptidoglycan was washed several times with water and recovered by microcentrifugation (11,000 × g, 20°C, 5 min). This material contained less than 0.02% phosphorus showing that over 98% of the original phosphorus had been removed.

Muramidase digestion. Each peptidoglycan sample was suspended in 0.5 ml of 30 mM triethanolamine acetate buffer (pH 4.7); *Chalaropsis muramidase B* (EC 3.2.1.17) (23) was added to a final concentration of 10 µg/ml, and the mixture was incubated at 37°C for 18 h. The enzyme was inactivated by heating to 100°C for 3 min, and insoluble material was removed by microcentrifugation. The supernatant, containing at least 95% of the original radioactivity, was lyophilized.

Fractionation of muropeptides by HPLC. The muropeptides obtained after digestion with *Chalaropsis muramidase B* (Fig. 1) were separated by high-performance liquid chromatography (HPLC) (System Gold; Beckman) as described previously (32). Samples (200 to 700 µg) were dissolved in 20 µl of water (HPLC grade; Rathburn) and injected onto a TSK2000 SW gel filtration column (7.5 mm by 60 cm; Beckman) fitted with a Spherogel TSK SW guard column (7.5 mm by 10 cm; Beckman). The muropeptides were separated in a running buffer of 50 mM sodium phosphate (pH 7.0) with a flow rate of 0.3 ml/min and detected at 214 nm. For the measurement of radioactivity, 4 ml of scintillant (Ready Safe; Beckman) was added to 90-µl samples collected from the HPLC column and counted.

Determination of the proportion of radiolabeled DAP in donor and acceptor positions of muropeptide dimers. Peptidoglycan was deaminated with nitrous acid (16), by suspending samples (~1 mg) in 0.8 ml of 0.5 M NaNO₂. Glacial acetic acid (130 µl) was added, the contents were mixed by vortexing, and the mixture was incubated at 0°C for 2 h. The samples were then treated with *Chalaropsis muramidase B*, and the muropeptides were fractionated by HPLC. The monomer and dimer fractions were hydrolyzed for 6 h at 100°C in 6 M HCl in evacuated thick-walled glass tubes. The acid was removed under diminished pressure, and the residue was suspended in water and dried (this step was repeated three times). Hydroxyaminopimelate and DAP were

separated by anion-exchange chromatography (16). Between 7 and 25% of the radioactivity in the monomer fraction remained as DAP after the deamination treatment, showing that this does not achieve complete conversion of unsubstituted amino groups. Similarly, incomplete conversion of DAP to hydroxyaminopimelic acid was also reported in work on *S. typhimurium* (9). For every sample, the conversion efficiency was determined for the monomer and the dimer result was adjusted accordingly.

RESULTS

The HPLC elution profile of digested peptidoglycan would be affected by incomplete hydrolysis of muramyl linkages with *Chalaropsis* muramidase. Thus a tetrasaccharide with two un-cross-linked peptides would have a molecular size similar to that of a disaccharide peptide dimer and would elute in the dimer peak. Complete cleavage of muramyl linkages was checked by an alkaline hydrolysis procedure (34). Monomer and dimer fractions were freeze-dried and then incubated in 4 M ammonium hydroxide (200 μ l) for 6 h at 25°C. The ammonia was removed under diminished pressure, and the samples were reexamined by HPLC. Under these conditions, a monomer is degraded to a lactyl peptide monomer and a modified disaccharide and a dimer is degraded to a lactyl peptide dimer and two molecules of the same modified disaccharide. An un-cross-linked tetrasaccharide peptide would give a lactyl peptide monomer and a modified tetrasaccharide peptide. When the degraded dimer was analyzed, no peak corresponding to lactyl peptide monomer was detected. However, peaks corresponding to lactyl peptide dimer and modified disaccharide were detected. This shows that the original dimer fraction was composed of *bis*-(disaccharide peptide).

The elution profile of the muropeptides, obtained after separation of the muramidase-digested peptidoglycan by gel filtration HPLC, is shown in Fig. 2. The percentage of cross-linking in the cell wall of *B. megaterium*, calculated (Appendix, equation 1) from the peak areas, was 53%. The distribution of the various disaccharide peptides in the cell wall is given in Table 1. The proportion of DAP residues in the wall with free amino groups was also determined by nitrous acid deamination (16). The corrected value obtained was 54%, in good agreement with the value obtained from the HPLC elution profile.

The changes in the distribution of radiolabeled DAP in muropeptides following pulsed incorporation are shown in Fig. 3, and the values at 30 s and 6 min are given in Table 2.

The labeling time of 30 s yielded dimers in which 94% (\pm 2%) of the radioactive DAP was in the donor position. The proportion of radioactive DAP in the donor position declined to just over 50% at 5 min after pulsed incorporation, concomitant with a rise in radioactivity in the acceptor position, and this value was maintained for all subsequent samples up to one generation time (Fig. 4). Muropeptide dimers isolated from bacteria that had been continuously labeled for several generations had 53% of their radioactivity in the donor position; this agreed well with the theoretical value of 50%. Five percent of the radioactivity was retained on the column when muropeptide dimers from peptidoglycan that had not been treated with nitrous acid were similarly analyzed. No correction was made for this effect.

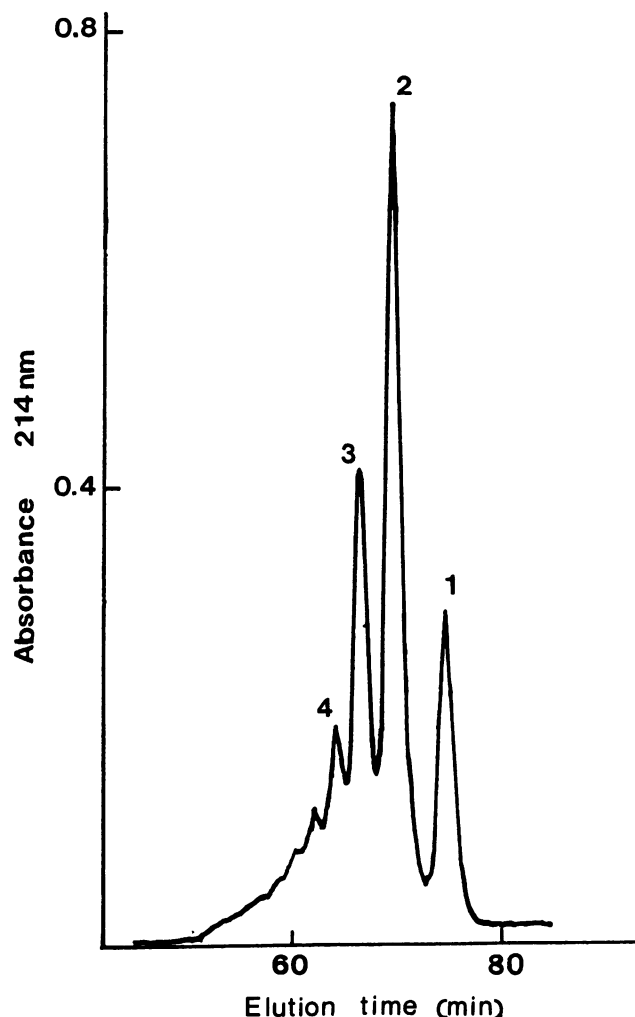


FIG. 2. Elution profile of muramidase-treated peptidoglycan from *B. megaterium*. Peptidoglycan, isolated as described in Results, was digested with *Chalaropsis* muramidase B, and the resulting muropeptides were separated by HPLC. The monomer, dimer, trimer, and tetramer peaks are labeled 1 to 4, respectively.

DISCUSSION

The results described in this paper have led to the formulation of a new model for the assembly of peptidoglycan in the cylindrical region of the cell wall of *B. megaterium*. This

TABLE 1. Distribution of muropeptides in peptidoglycan of *B. megaterium* and theoretical distributions derived from the equations for monomer and random addition polymerization reactions

Distribution	Proportion of muropeptides (% of total peptidoglycan)				
	Monomer	Dimer	Trimer	Tetramer	Pentamer
Observed	14.6	42.6	22.0	9.5	4.9 ^a
Monomer addition	15.2	34.3	29.0	14.5	5.1
Random addition	22.1	23.4	18.6	13.1	8.7

^a It was not possible to determine accurately the distribution of individual oligomers beyond the pentamer. Higher oligomers accounted for approximately 7% of the peptidoglycan.

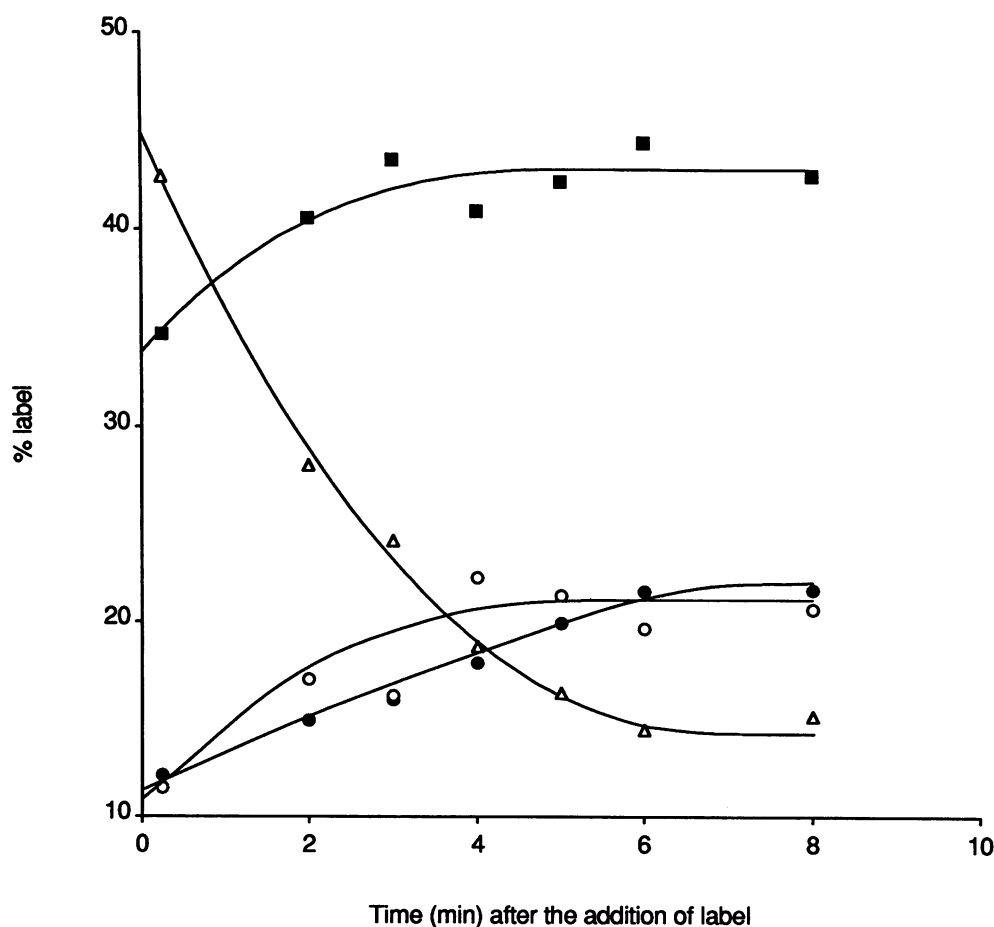


FIG. 3. Change in the distribution of labeled muropeptides at various times following the addition of [^3H]DAP. Δ , Monomer; \blacksquare , dimer; \bullet , trimer; \circ , higher oligomers.

model may also apply to other gram-positive bacilli and, in modified form, to gram-negative bacilli. We propose the following. (i) Single nascent glycan peptide strands are incorporated into the existing wall by transpeptidation reactions in which the incoming peptides act as donors in forming cross-linkages with material located at the inner surface of the wall. (ii) Cross-linking takes place only at the inner surface of the wall and only at the time that incoming new material is incorporated. Maturation of cross-linking is a direct consequence of subsequent incorporation of further new material, and there is no separate secondary cross-linking process. (iii) The pattern of cross-link formation by the new glycan peptide monomers is always the same, and, consequently, the initial distribution of muropeptide monomers, dimers, and trimers, etc., is constant. It follows that the final pattern of cross-linking in the wall is determined

TABLE 2. Distribution of radioactivity in muropeptides following the pulsed incorporation of [^3H]DAP into the peptidoglycan of *B. megaterium*

Time	% Radioactivity in isolated muropeptides			
	Monomer	Dimer	Trimer	Higher oligomer
30 s	43	35	12	10
6 min	14	43	22	21

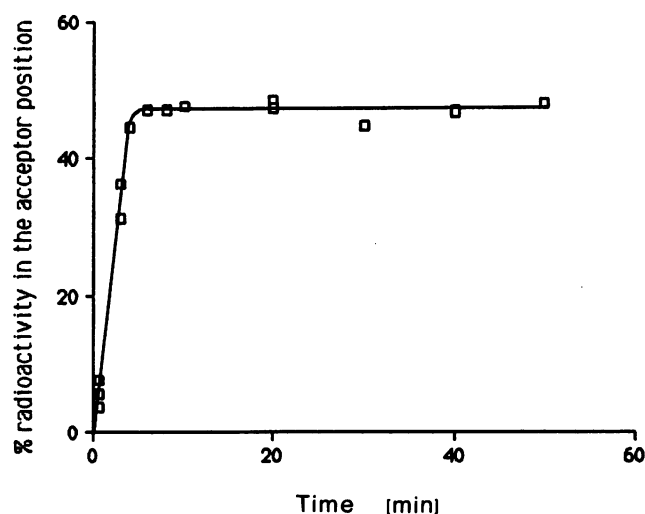


FIG. 4. Proportion of radioactive DAP in the acceptor position of isolated muropeptide dimers at various intervals after addition of a radioactive pulse of [^3H]DAP.

solely by, and can be forecast from, the repetitive pattern of incorporation of new material. (iv) The initial pattern is maintained by a set of constant probabilities governing the formation of the different mucopeptides. These probabilities may reflect both enzymic and steric effects.

The present experimental observations on *B. megaterium* are fully consistent with this model.

The efficiency of the DAP uptake system of *B. megaterium* KM allowed the use of a short, well-defined pulse of radioactivity (17). In samples collected immediately after 30 s incubation with radiolabeled DAP, almost all (94%) of the radiolabel was in the donor position of isolated dimers, confirming the earlier report that incoming peptides act as donors in forming cross-links with the growing cell wall (16). Very little radioactive DAP was present in the acceptor position in these dimers, showing that the incoming labeled peptidoglycan chains had been linked only to existing wall and not to each other. Such single-strand incorporation has also been shown in *S. typhimurium* (9). An earlier conclusion that incorporation in *E. coli* proceeded by double-strand insertion was based on the finding that a substantial proportion of newly incorporated DAP occupied the acceptor position (5). However, this observation was found subsequently to be the result of septal murein synthesis (29), and it now appears that both gram-negative and gram-positive bacilli use a single-strand mode of peptidoglycan incorporation for cylindrical wall growth.

Following the DAP pulse, the amount of radioactivity in the acceptor position of the dimer fraction increased rapidly to a value of 48% by 6 min (Fig. 4). This value remained stable for up to 50 min (1.2 generation times) after the pulse. The distribution of radioactivity between the mucopeptides stabilized within 6 min of the addition of the pulse, showing that little or no further cross-linking to the radioactive fragments was occurring after this time.

The amounts of wall material synthesized can be calculated from the relation $w_t = w_0 e^{\mu t}$ (2), where w_0 and w_t are the amounts of wall material present initially and at time t and μ is the specific growth rate constant. During the 30-s labeling period, 0.84% more wall is synthesized; this increases to 10.5% after 6 min of growth. Since cross-linking is complete in 6 min, it follows that cross-linking takes place only in the innermost region of the wall.

By the time cross-linking is complete, the same numbers of dimers will have been formed by the addition of incoming unlabeled monomers to labeled monomer as had been formed when the latter were the incoming monomers (Fig. 5). Therefore, there should be an equal distribution of labeled DAP in the donor and acceptor positions of the dimer. The experimental value of 48% of label in the acceptor position is consistent with this premise.

A consequence of the model is that the final pattern of cross-linking in the wall can be predicted from the initial distribution of labeled mucopeptides. As shown in Fig. 5, an initial distribution in which 60% of the incoming peptides remain as monomers, 30% are cross-linked to form dimers, and 10% are cross-linked to form trimers gives mature layers (layers 3 and above) that have 30% of their peptides present as monomers, 40% as one of the two units of a dimer, and 30% as one of the three units of a trimer. It follows that in the mature wall the molar proportion of monomers:dimers:trimers is 3:2:1. It may be noted that this molar distribution can be obtained directly from the initial distribution as the final number of " n -mers" is equal to the difference between the initial numbers of n -mers and $(n + 1)$ -mers. For example in Fig. 5 the initial distribution of 6 monomers to 3 dimers to

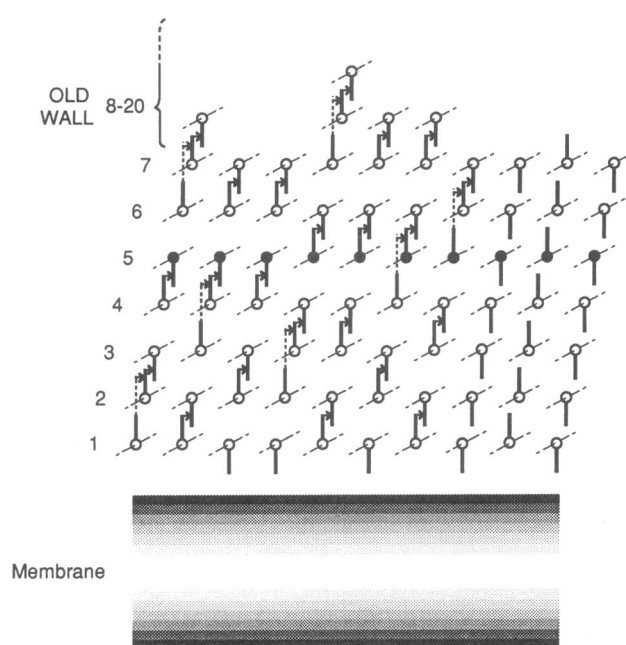


FIG. 5. Schematic diagram to illustrate the pattern of cross-linking in a growing cell wall. Layer 1 shows a pattern of incorporation of new material in which, for every 10 peptide monomers incorporated, 6 remain as monomers, 3 cross-link to existing wall monomers to form dimers, and 1 cross-links to an existing wall dimer to form a trimer. When a further layer of material is incorporated, using the same pattern of incorporation, layer 1 moves up to become the second layer. Three of the original six monomers become converted to dimers, and one of the original three dimers becomes a trimer. Consequently, the layer now contains three monomers and peptides forming part of five dimers and two trimers. When the next layer is incorporated, the layer moves up to become the third layer and in the process one of its dimers becomes a trimer, so that the now-mature layer contains three monomers and peptides forming part of four dimers and three trimers. Further incorporation of new material has no effect on the pattern of cross-linking involving this layer, and its pattern of cross-linking reflects that of the mature cell wall, as illustrated in the diagram by a layer (layer 5) of pulse-labeled glycan peptides. To enable the process to be clearly depicted, cross-linkages are only shown perpendicular to glycan sheets. In the wall, however, peptides will radiate from the helically twisted glycan chain and so will connect chains at various angles; these chains are unlikely to be arranged into regular sheets as indicated for simplicity here. ○ Glycan strand; ● pulse-labeled layer; ■ peptide.

1 trimer leads to a final distribution of 3 (i.e., $6 - 3$) monomers to 2 (i.e., $3 - 1$) dimers to 1 (i.e., $1 - 0$) trimer. Applying this to the initial distribution of labeled mucopeptides in *B. megaterium* (Table 2) gives a predicted molar distribution (Table 3, prediction A) of mucopeptides that compares well with that observed. The initial dimer contained 6% of the label in the acceptor position. This could be due to the formation of a small proportion of dimers from labeled monomers that had already acted as acceptors. When the initial distribution was corrected for this effect, the prediction (Table 3, prediction B) was even closer to the experimental value.

The close agreement between the observed and calculated final distributions of mucopeptides supports the proposal that the formation of cross-links during the assembly of the cell wall of *B. megaterium* takes place only during incorpo-

TABLE 3. Predicted and observed molar distributions of muropeptides in the peptidoglycan of *B. megaterium*

Distribution	Muropeptides		
	Monomer	Dimer	Trimer
Observed ^a	14.6	21.3	7.3
Prediction A ^b	8.0	23.0	6.5
Prediction B ^c	12.0	21.0	6.5

^a Calculated by dividing the values shown in Table 1 by the number of disaccharide peptide monomer units in each particular muropeptide, e.g., dividing the dimer value by 2 and the trimer value by 3.

^b Calculated from the uncorrected distribution at 30 s as described in the text: i.e., predicted monomer = monomer (30 s) minus dimer (30 s); predicted dimer = dimer (30 s) minus trimer (30 s), etc.

^c Calculated as in footnote *b* from the distribution at 30 s, corrected as indicated in the text.

ration and there is no requirement for a subsequent secondary cross-linking process. Such a monomer addition process should lead to a different distribution of disaccharide peptide oligomers than that resulting from a secondary cross-linking process.

Oldmixon and colleagues (28) have applied the equations (Appendix) derived by Flory (15) for monomer and random polymerization in solution to predict the distribution of muropeptides that would result from these two processes. The equations use a single variable, namely the degree of cross-linking (see Appendix). When the cross-linking value of *B. megaterium* is applied to these equations, the distributions shown in Table 1 are obtained. The distribution obtained experimentally does not obviously fit either prediction, nor is it a combination of the two, but it is closer to that predicted by a monomer addition model, as might be expected from a single-stranded mode of incorporation. The distribution of muropeptides in *Streptococcus faecalis* (28) was also closer to that expected of a monomer addition mechanism. A similar analysis of *Staphylococcus aureus* gives results that are in accord with an overall random polymerization process, possibly involving an initial stage of monomer addition followed by secondary random cross-linking (31). However, it is not obvious that polymerization equations derived for homogeneous catalysis in solution can be legitimately applied to the vectorial assembly of the cell wall. The kinetic analysis of monomer addition (15) required that all oligomers retain an active terminus throughout the polymerization and that all active termini are equally susceptible to reaction with monomer. It is unlikely that these conditions are met during cell wall synthesis, since if cross-linking takes place only at the inner surface it cannot involve oligomers in the outer region of the wall. If an incoming monomer peptide had an equal probability of cross-linking to a monomer or to a dimer, then the ratio of dimer:trimer at 30 s should be the same as the ratio of monomer:dimer available at the inner surface of the wall. Oldmixon et al. (28) postulated that the distribution at the inner surface of the wall would be the same as in the wall overall. On this basis, the molar ratio monomer:dimer (Table 3) should lead to the formation of new dimer:trimer in the same ratio (0.7:1). This is substantially different from that obtained experimentally (2.9:1). The observed distribution contains more dimer and less trimer than that predicted. As indicated by our results and shown in the model (Fig. 5), the distribution of muropeptides at the inner surface of the cell wall will not be the same as in the wall overall but will be that of the newly inserted peptidoglycan, i.e., similar to that found after 30 s of labeling

(Table 2). By using these values, the predicted dimer:trimer ratio (1.2:1) is still less than that observed (2.9:1) although closer to it than was the ratio originally predicted on the basis of the work of Oldmixon et al. (28). The results indicate a bias in the ability of incoming monomer peptide to cross-link to a monomer as opposed to a dimer already incorporated into the inner surface of the wall. The changes observed in the distribution of labeled DAP in muropeptides between 30 s and 6 min (beyond which time no further cross-linking involving labeled units was detected) demonstrate this point (Table 2). During this time, the proportion of labeled DAP in monomer falls from 43 units (100% = 100 units) to 14 units. Therefore, the chance of a monomer in the wall accepting an incoming monomer and forming a dimer is 29/43, i.e., 0.67. Over the same period, only 21 units of labeled dimer, from a total of 64 (the new 29 units plus the original 35 units), act as acceptors to incoming monomers, a probability of 21/64, i.e., 0.33.

This bias can be explained by steric effects: incoming monomer peptides and potential acceptor peptides may form cross-linkages only when they are in appropriate steric dispositions (4). Incoming peptides oriented roughly parallel to the plane of the wall or projecting into that plane may be able to form cross-links with appropriately disposed wall peptides, but incoming peptides oriented inwards away from the plane of the wall may not. The incoming peptides may link only to those wall peptides that are near its inner surface and that are oriented roughly in the plane of the wall or that project inwards from it. A wall peptide involved in a dimer will rarely project inwards from the plane of the wall and, consequently, is less likely to be in an appropriate orientation for accepting an incoming monomer than is a wall peptide that had not formed a cross-link during its incorporation. Therefore, the probability that a peptide involved in a dimer will form a further cross-link (i.e., to form a trimer) will be less than that of a monomer forming a cross-link (i.e., to form a dimer). The proportion of dimer in the wall will therefore be higher than that expected if the peptides in the inner surface of the wall are equally likely to accept incoming monomers.

Our results are consistent with a monomer addition model in which a monomer in the inner wall is approximately twice as likely to accept incoming peptidoglycan as is a peptide already involved in a cross-linkage (i.e., dimer, trimer, etc.).

Peptidoglycan assembly in *E. coli* may proceed in a manner similar to that proposed for *B. megaterium*. New material is cross-linked to the underside of the existing wall by a monomer addition process. In one variant of the model, the new glycan strand is cross-linked at different sites to two adjacent glycan strands above it. Subsequently, the peptide cross-linkages directly connecting those two adjacent strands are cleaved and the new glycan strand becomes part of the stress-bearing wall. In another variant of this model, a second new strand is incorporated alongside, and cross-linked to, the first before the overlying peptides are cleaved. The two new strands are also linked to three adjacent overlying strands, cleavage of which can result in the release of the middle strand. This permits turnover of wall material, as observed in *E. coli* (21).

Newly incorporated labeled DAP is present mainly in monomers and in the donor peptide of dimers. As further material is incorporated, some existing labeled monomers act as acceptors, so increasing the proportion of labeled DAP in acceptor peptides. The labeled dimers originally formed will be cleaved as the wall expands, so reducing the amount of labeled DAP in donor peptides. This can account

for the observed changes in the location of labeled DAP in *E. coli* (6, 20) and, together with the reincorporation of turned-over material (19, 21), for the observation that the amount of label in cross-linkages reached a constant value after a prolonged labeling period.

The models discussed here relate to the growth of cylindrical wall in rod-shaped bacteria. Wall assembly during septation may proceed differently but could still involve a monomer addition process. A reduction in the time between the incorporation of a peptide monomer and its subsequent participation as an acceptor could account for the relatively high initial acceptor values found in constricting cultures of *E. coli* (10). In gram-positive bacilli there are differences between polar and cylindrical wall with respect to turnover (8) and susceptibility to autolysins (14) but little is known about differences in their composition and their mechanisms of assembly.

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APPENDIX

The percentage of cross-linking in peptidoglycan can be determined (26) from the muropeptide composition expressed as percentage (wt/wt) of peptidoglycan.

$$\% \text{ cross-linking} = [1/2 \times (\% \text{ dimer})] + [2/3 \times (\% \text{ trimer})] + [3/4 \times (\% \text{ tetramer})] + \dots + [(n-1)/n \times (\% n\text{-mer})] \quad (1)$$

where n is the number of disaccharide peptide monomer units in a particular muropeptide.

The following equations were derived by Flory (15) for monomer and random polymerization in solution and applied by Oldmixon et al. (28) to predict the distribution of muropeptides in the cell wall that would result from these two processes.

Monomer addition:

$$\% n\text{-mer} = \frac{nv^{n-1}}{(v+1)e^{v(n-1)}} \times 100 \quad (2)$$

Random addition:

$$\% n\text{-mer} = np^{n-1}(1-p)^2 \times 100 \quad (3)$$

where $v = [1/(1-p)] - 1$, n = number of disaccharide peptides in the muropeptide, and p = the fraction of DAP residues that are cross-linked.

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